Table 1 Characteristics of patients with type 2 diabetes stratified by albuminuria status

	Microalbuminuria	*Normoalbuminuria*	*p*
Urinary albumin (mg/gCr)	90 ± 8	10 ± 1	0.001
Number of subjects	45	43	
M/F	29/16	34/9	0.159
Age (yrs)	65.6 ± 1.2	59.8 ± 1.3	0.001
Systolic blood pressure (mm Hg)	140 ± 3	133 ± 3	0.940
Diastolic blood pressure (mm Hg)	81 ± 2	83 ± 2	0.570
Smoking (yes/no)	9/36	13/30	0.328
Duration of diabetes (yrs)	11.7 ± 1.1	10.2 ± 1.1	0.314
BMI (kg/m^2)	22.6 ± 0.3	23.1 ± 0.3	0.301
Fasting glucose (mg/dl)	144 ± 3	138 ± 4	0.256
HbA_{1c} (%)	7.1 ± 0.1	7.0 ± 0.2	0.654
Fasting insulin (μU/ml)	6.6 ± 0.6	6.5 ± 0.4	0.540
Triglycerides (mg/dl)	119 ± 8	124 ± 9	0.705
Total cholesterol (mg/dl)	204 ± 6	204 ± 5	0.929
HDL cholesterol (mg/dl)	58 ± 2	59 ± 2	0.715
LDL cholesterol (mg/dl)	126 ± 5	126 ± 5	0.984
Serum creatinine (mg/dl)	0.77 ± 0.03	0.75 ± 0.02	0.537
TNF-α (ng/l)	3.5 ± 0.4	3.2 ± 0.2	0.479
sTNF-R1 (ng/l)	1272 ± 71	1084 ± 33	0.018
sTNF-R2 (ng/ml)	2172 ± 91	1933 ± 49	0.022
SU/diet	40/5	35/8	0.094
HMG-CoA reductase inhibitor	7/38	5/38	0.284
Bezafibrate	7/38	12/31	0.090
Ca antagonist	11/34	7/36	0.147
ACE inhibitor or ARB	8/37	8/35	0.500

Results

The clinical characteristics and clinical profile between the patients with microalbuminuria (n = 45) and normoalbuminuria (n = 43) were compared (Table **1**). Urinary albumin concentrations in patients with microalbuminuria and normoalbuminuria were 90 ± 8 (range, 35 – 282) and 10 ± 1 (range, 0.6 – 24.9) mg/g creatinine, respectively. There was no overlap in the urinary concentration of albumin between the two groups. While age was significantly greater in the patients with microalbuminuria than those with normoalbuminuria, no significant difference was observed in systolic and diastolic blood pressure, smoking, diabetes duration, BMI, fasting glucose, hemoglobin A_{1c}, or fasting insulin levels between the two groups. The two groups did not differ with respect to concentrations of serum triglycerides, total, HDL, or LDL cholesterol. Although there was no significant difference in the levels of serum creatinine and TNF-α, soluble TNF-R1 (1,272 ± 71 *vs.* 1,084 ± 33 pg/ml, p = 0.018), and soluble TNF-R2 (2172 ± 91 *vs.* 1933 ± 49 pg/ml, p = 0.022) were significantly higher in patients with microalbuminuria compared to those with normoalbuminuria.

Spearman's rank correlations of urinary albumin concentration with measures of variables were calculated for all our diabetic patients (Table **2**). Urinary albumin concentration was positively correlated with soluble TNF-R1 (r = 0.364, p < 0.001), soluble TNF-R2 (r = 0.342, p < 0.005), age (r = 0.380, p < 0.001), and serum creatinine (r = 0.214, p < 0.05). Other variables including systolic and diastolic blood pressure, TNF-α, and serum lipid profile including triglycerides were not associated with urinary albumin level. Multiple regression analyses were carried out using the stepwise procedure.

The analysis included urinary albumin level as a dependent variable and candidate risk factors (soluble TNF-R1, soluble TNF-R2, age, serum creatinine) as independent variables (Table **2**). The concentration of urinary albumin was independently predicted by serum concentration of soluble TNF-R1, which explained 26.3 % of the variability of urinary albumin concentration in our patients. Other variables including age, serum creatinine, and soluble TNF-R2 were not independently associated with urinary albumin concentration in our non-obese Japanese type 2 diabetic patients. On the other hand, in a model incorporating BMI and systolic blood pressure, soluble TNF-R1 was also independently associated with urinary albumin concentration in our patients (Table **3**).

Discussion and Conclusions

This is the first published observation that soluble TNF-R1 is independently associated with urinary albumin concentration in non-obese Japanese type 2 diabetic patients.

Diabetic nephropathy has rapidly become an important public health problem since it is the leading cause of dialysis in Japan. Early detection of risk factors causing diabetic nephropathy before advanced renal damage occurs is therefore an urgent prior-

Table 2 Correlation of urinary albumin concentration to measures for variables in diabetic patients

	Univariate		*Multivariate*
	r	*p*	*F*
TNF-α	0.127	0.236	–
sTNF-R1	0.364	< 0.001	32.1
sTNF-R2	0.342	< 0.005	0.2
Age	0.380	< 0.001	1.9
Serum creatinine	0.214	0.046	0.1
Gender	– 0.083	0.440	–
Diabetes duration	0.202	0.060	–
BMI	– 0.191	0.076	–
Systolic blood pressure	0.189	0.097	–
Diastolic blood pressure	– 0.079	0.488	–
Fasting glucose	0.104	0.334	–
$HbA1_c$	0.136	0.203	–
Triglycerides	– 0.081	0.452	–
Total cholesterol	– 0.077	0.471	–
HDL cholesterol	– 0.109	0.310	–
LDL cholesterol	– 0.094	0.383	–

Table 3 Determinants of urinary albumin concentration by multivariate analysis

	Model 1 (F)	*Model 2 (F)*
sTNF-R1	32.1	31.0
sTNF-R2	0.2	0.2
Age	1.9	0.7
Serum creatinine	0.1	0.2
BMI	–	0.1
Systolic blood pressure	–	1.8
R2	0.263	0.280

ity. Microalbuminuria has been shown to be not only an indicator of incipient nephropathy but also an independent risk factor for cardiovascular disease [5]. The mechanisms underlying the evolution of microalbuminuria in diabetic patients are not fully clarified. Genetic factors, insulin resistance, glycemic control, blood pressure, smoking, and lipid abnormalities have been implicated in albuminuria development in diabetic patients [23].

Inflammation seems to be associated with urinary albumin excretion in diabetic patients. Gabazza et al. [24] showed high concentrations of serum fibrinogen in type 2 diabetic patients with albuminuria compared to those without albuminuria. Microalbuminuria has been shown to be associated with fibronectin and sialic acid in type 2 diabetic patients [25,26]. Furthermore, Festa et al. [6] have reported an association of CRP and fibrinogens with urinary albumin excretion in the microalbuminuric range of type 2 diabetic individuals. Stehouwer et al. [7] confirmed that increased urinary albumin excretion, endothelial dysfunction, and chronic inflammation are interrelated processes associated with risk of death in type 2 diabetic patients.

In the present study, we investigated the relationship between albuminuria and TNF-α system after carefully matching the participants for smoking, BMI, blood pressure, glycemic control, and lipid profile. We used serum TNF-α, soluble TNF-R1, and soluble TNF-R2 as the index of TNF-α system activity and found, for the first time, that soluble TNF-R1 was independently associated with albuminuria in type 2 diabetic patients. However, we could not find the relationship between albuminuria and TNF-α. The reason is not known, but may be due to circulating TNF receptor levels remaining elevated for a longer time than TNF-α itself and reflecting the degree of TNF-α activation more accurately than the measurement of TNF-α itself. TNF receptor levels might be considered to be a more valuable factor for monitoring the degree of TNF-α system activity. Thus, the TNF-α system could predispose to the development of microalbuminuria in type 2 diabetic patients. Baud and Ardaillou [27] have shown that TNF-α induces glomerular infiltration by leukocytes. Klein et al. [28] have demonstrated that TNF-α influences the metabolism of glycosaminoglycans, which are components of the vascular endothelium and the glomerular basement membrane and are involved in the etiology of microalbuminuria.

The mechanisms for the increased activity of TNF-α system in type 2 diabetic patients with microalbuminuria are unknown; however, elevated synthesis, reduced catabolism, or both must be present. *In vitro* investigations have shown increased TNF-α messenger RNA expression in glomeruli from diabetic rats [29]. Recent studies have demonstrated that advanced glycation end products binding to specific cell-surface receptor molecules expressed on kidney cells may induce local cytokine and initiate local inflammatory reaction [30]. Angiotensin II, a substance associated with development of renal injury in diabetic patients, has been shown to upregulate TNF-α expression [31].

Interestingly, soluble TNF-R1, but not soluble TNF-R2, was associated with albuminuria in our diabetic patients. The reasons for the discrepancy between the TNF-R1 or TNF-R2 relationship to albuminuria in our diabetic patients are not clear. These two receptors seem to differ in terms of signaling and functional properties [8–10]. Several studies have demonstrated that obese subjects overexpress TNF-alpha and TNF-R2 in adipose tissue and have higher concentrations of serum TNF-R2 levels in relation to lean controls [32,33]. TNF-α can upregulate TNF-R2 expression in humans [34]. In contrast, the majority of biological responses classically attributed to TNF-α such as cytotoxicity and nuclear kappa B activation are mediated by TNF-R1 [35]. Pichler et al. [36] have shown that sTNF-R1 may play an important role in the onset of the acute stage of Graves' disease.

Nevertheless, the present study that TNF-R1, but not TNF-R2, is associated with albuminuria in diabetic patients suggests that TNF-R1 may play a role in the evolution of vascular complications in our non-obese type 2 diabetic patients. This idea is supported by the recent study by Rauchhaus et al. [11] demonstrating that elevated soluble TNF-R1 levels are predictive of cardiovascular mortality in patients with chronic heart failure. Furthermore, Zoppini et al. [12] have reported that TNF-R1 is associated with the progression of microalbuminuria and retinopathy in type 1 diabetic patients.

In summary, although our present study was performed among the limited patients that were well-controlled in terms of BMI, HbA_{1c}, blood pressure, LDL cholesterol, triglycerides, total and HDL cholesterol, serum soluble TNF-R1 seems to be associated with albuminuria in non-obese Japanese type 2 diabetic patients. Further study should be undertaken to clarify whether or not serum soluble TNF-R1 is reflective of early stage of atherosclerosis in non-obese Japanese type 2 diabetic patients.

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References

[1] Fuller H, Shipley MJ, Rose G, Jarrett RJ, Keen H. Coronary heart disease risk and impaired glucose tolerance. Lancet 1980; 1: 1373 – 1376

[2] Yano K, Kagan A, McGee D, Rhoads GG. Glucose intolerance and nine-year mortality in Japanese men in Hawaii. Am J Med 1982; 72: 71 – 80

[3] Diabetes Drafting Group. Prevalence of small vessel and large vessel disease in diabetic persons from 14 centers: the World Health Organization multinational study of vascular disease in diabetics. Diabetologia 1985; 28: 615 – 640

[4] Herman JB, Medalie JH, Goldbourt U. Differences in cardiovascular morbidity and mortality between previously known and newly diagnosed adult diabetics. Diabetologia 1977; 13: 229 – 234

[5] Mogensen CE. Microalbuminuria predicts clinical proteinuria and early mortality in maturity-onset diabetes. N Engl J Med 1984; 310: 356 – 360

[6] Festa A, D'Agostino R, Howard G, Mykkanen L, Tracy RP, Haffner SM. Inflammation and microalbuminuria in nondiabetic and type 2 diabetic subjects: The insulin resistance atherosclerosis study. Kidney Int 2000; 58: 1703 – 1710

[7] Stehouwer CDA, Gall MA, Twisk JWR, Knudsen E, Emeis JJ, Parving HH. Increased urinary albumin excretion, endothelial dysfunction, and chronic low-grade inflammation in type 2 diabetes. Progressive, interrelated, and independently associated with risk of death. Diabetes 2002; 51: 1157 – 1165

[8] Smith CA, Farrah T, Goodwin RG. TNF receptor superfamily of cellular and viral proteins: activation, costimulation and death. Cell 1994; 76: 959 – 962

[9] Tartaglia LA, Weber RF, Figari IS, Reynolds S, Pailadino MA Jr, Goeddel DV. The two different receptors. Proc Natl Acad Sci USA 1991; 88: 9292 – 9296

[10] Tartaglia LA, Goeddel DV. Two TNF receptors. Immunol Today 1992; 13: 151 – 153

[11] Rauchhaus M, Doehner W, Francis DP, Davos C, Kemp M, Liebenthal C, Niebauer J, Hooper J, Volk HD, Coats AJS, Anker SD. Plasma cytokine parameters and mortality in patients with chronic heart failure. Circulation 2000; 102: 3060 – 3067

[12] Zoppini G, Faccini G, Muggeo M, Zenari L, Falezza G, Targher G. Elevated plasma levels of soluble receptors of TNF-α and their association with smoking and microvascular complications in young adults with type 1 diabetes. J Clin Endocrinol Metab 2001; 186: 3805 – 3808

[13] Hotamisligil GS, Shargil NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 1993; 259: 87 – 91

[14] Tanaka S, Seino H, Satoh J, Fujii N, Rikiishi H, Zhu XP, Takahashi K, Sagara M, Nobunaga T, Toyota T. Increased in vivo production of tumor necrosis factor after development of diabetes in non-treated, long-term diabetic BB rats. Clin Immunol Immunopathol 1992; 62: 258 – 263

[15] Taniguchi A, Nakai Y, Doi K, Fukuzawa H, Fukushima M, Kawamura H, Tokuyama K, Suzuki M, Fujitani J, Tanaka H, Nagata I. Insulin sensitivity, insulin secretion, and glucose effectiveness in obese subjects: a minimal model analysis. Metabolism 1995; 44: 1397 – 1400

[16] World Health Organization. Diabetes Mellitus: Report of a WHO Study Group. Geneva: World Health Org, 1985; (Tech. Rep. Ser., no 727)

[17] Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972; 18: 499 – 502

[18] Nakai Y, Hamagaki S, Seino Y, Takagi R, Taniguchi A, Kurimoto F. Plasma concentrations of tumor necrosis factor-α (TNF-α) and soluble TNF receptors in patients with anorexia nervosa. J Clin Endocrinol Metab 1999; 84: 1226 – 1228

[19] Gatling W, Knight C, Hill RD. Screening for early diabetic nephropathy: which sample to detect microalbuminuria? Diabetic Med 1985; 2: 451 – 455

[20] Cowell CT, Rodgers S, Silkink M. First morning urinary albumin concentration is a good predictor of 24-hour urinary albumin excretion in children with type 1 (insulin-dependent) diabetes. Diabetologia 1986; 29: 97 – 99

[21] Hutchison AS, O'Reilly DSJ, McCuish AC. Albumin excretion rate, albumin concentration, and albumin/creatinine ratio compared for screening diabetics for slight albuminuria. Clin Chem 1988; 34: 2019 – 2021

[22] American Diabetes Associations. Nephropathy in diabetes. Diabetes Care 2004; 27: 579 – 583

[23] Groop L, Ekstrand A, Forsblom C, Widen E, Groop PH, Teppo AM, Eriksson J. Insulin resistance, hypertension, and microalbuminuria in patients with type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia 1993; 36: 642 – 647

[24] Gabazza EC, Takeya H, Debuchi H, Sumida Y, Taguchi O, Murata K, Nakatani K, Yano Y, Mohri M, Sata M, Shima T, Nishioka J, Suzuki K. Protein C activation in NIDDM patients. Diabetologia 1996; 39: 1455 – 1461

[25] Nielsen S, Schmitz A, Bacher T, Rehling M, Ingerslev J, Mogensen CE. Transcapillary escape rate and albuminuria in type II diabetes: Effects of short-term treatment with low-molecular weight heparin. Diabetologia 1999; 42: 60 – 67

[26] Chen JW, Gall MA, Yokoyama H, Jensen JS, Deckert M, Parving HH. Raised serum sialic acid concentration in NIDDM patients with and without diabetic nephropathy. Diabetes Care 1996; 19: 130 – 134

[27] Baud L, Ardaillou R. Tumor necrosis factor alpha in glomerular injury. Kidney Int 1994; 45 (Suppl 45): S32 – S36

[28] Klein NJ, Shennan GI, Heyderman RS, Levin M. Alteration in glycosaminoglycan metabolism and surface charge on human umbilical vein endothelial cells induced by cytokines, endotoxin, and neutrophils. J Cell Sci 1992; 102: 821 – 832

[29] Nakamura T, Fukui M, Ebihara I, Osada S, Nagaoka I, Tomino Y, Koide H. mRNA expression of growth factors in glomeruli from diabetic rats. Diabetes 1993; 42: 450 – 456

[30] Basta G, Lazzerini G, Massaro M, Simoncini T, Tanganelli P, Fu C, Kislinger T, Stern DM, Schmidt AM, De Caterina R. Advanced glycation end products activate endothelium through signal-transduction receptor RAGE. A mechanism for amplification of inflammatory responses. Circulation 2002; 105: 816 – 822

[31] Ruits-Ortega M, Lorenzo O, Suzuki Y, Ruperez M, Egido J. Proinflammatory actions of angiotensins. Curr Opin Nephrol Hypertens 2001; 10: 321 – 329

[32] Hotamisligil GS, Arner P, Atkinson RL, Spiegelman M. Differential regulation of the p80 tumor necrosis factor receptor in human obesity and insulin resistance. Diabetes 1997; 46: 451 – 455

[33] Fernandez-Real JM, Broch M, Ricart W, Casmitjana R, Gutierrez C, Vendrell J, Richart C. Plasma levels of the soluble fraction of tumor necrosis factor 2 and insulin resistance. Diabetes 1998; 47: 1757 – 1762

[34] Liu LS, Spelleken M, Rohrig K, Hauner H, Eckel J. Tumor necrosis factor-α acutely inhibits insulin signaling in human adipocytes. Implication of the p80 tumor necrosis factor receptor. Diabetes 1998; 47: 515 – 522

[35] Vandenabeele P, Declercq W, Beyaert R, Fiers W. Two tumor necrosis factor receptors: structure and function. Trends in Cell Bilogy 1995; 5: 392 – 399

[36] Pichler R, Maschek W, Hatzl-Griesenhofer M, Huber H, Crespillo-Gomez C, Berg J. Soluble tumor necrosis factor-α receptor 1 and interleukin-6 as markers of activity in thyrotoxic Graves' disease. Horm Metab Res 2003; 35: 427 – 433

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Three measures of tumor necrosis factor α activity and insulin resistance in nonobese Japanese type 2 diabetic patients

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Abstract

The aim of the present study was to investigate the relationship between insulin resistance and tumor necrosis factor α (TNF-α) as well as soluble TNF receptors (sTNF-R), body mass index (BMI), leptin, adiponectin, and serum lipid profile including triglycerides in nonobese Japanese patients with type 2 diabetes. A total of 88 nonobese Japanese type 2 diabetic patients were studied. The duration of diabetes was 11.0 ± 0.8 years. In conjunction with BMI, glycosylated hemoglobin (HbA1c), fasting concentrations of plasma glucose, serum lipids (triglycerides, high-density lipoprotein cholesterol, and total cholesterol), serum leptin, serum adiponectin, serum TNF-α, and soluble TNF receptors (sTNF-R1 and sTNF-R2) were also measured. Insulin resistance was estimated by the insulin resistance index of homeostasis model assessment. Insulin resistance was positively correlated with BMI, triglycerides, leptin, and total cholesterol and negatively correlated with adiponectin and high-density lipoprotein cholesterol. In contrast, insulin resistance was not associated with TNF-α, nor sTNF-R (sTNF-R1 and sTNF-R2) in our diabetic patients. There was no significant relationship between the 3 measures of TNF-α system (TNF-α, sTNF-R1, and sTNF-R2) and BMI, serum triglycerides, leptin, or adiponectin in these patients. From these results, it can be concluded that peripheral levels of TNF-α system activity are not a major factor responsible for insulin resistance in nonobese Japanese type 2 diabetic patients.

1. Introduction

Type 2 diabetes mellitus is a heterogeneous syndrome characterized by insulin resistance and/or defective insulin secretion [1]. In contrast to white populations, nonobese Japanese patients with type 2 diabetes are unique in that they are divided into 2 variants: one with insulin resistance and the other with normal insulin sensitivity [2-9]. The former group is characterized by higher body mass index (BMI), higher triglycerides, higher leptin, and lower adiponectin as compared with the latter group. Whereas serum leptin level is shown to be associated with subcutaneous fat area, serum concentrations of triglycerides and adiponectin are linked to visceral fat areas in nonobese Japanese type 2 diabetic patients [7-9]. Thus, the adipose tissue–linked substances are hypothesized to be associated with insulin resistance in nonobese Japanese type 2 diabetic patients.

Tumor necrosis factor α (TNF-α) is 1 of the most important candidates expressed in human adipocytes [10]. Adipocytes of obese subjects are reported to have higher rates in TNF-α messenger RNA expression and TNF-α protein production as compared with those of nonobese subjects, thus resulting in a greater serum TNF-α concentration in obese subjects [11-13]. The increase in TNF-α messenger RNA levels is positively correlated to the degree

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of hyperinsulinemia, and weight loss is accompanied by a decrease in serum TNF-α concentration and an increase in insulin sensitivity [11-13]. It remains, however, unsolved whether the relationships between serum TNF-α and insulin resistance are caused by or are a result of obesity itself. Furthermore, it is suggested that glucose is proinflammatory and may potentially induce TNF-α. To address this, we recruited nonobese well-controlled Japanese type 2 diabetic patients carefully stratified by their resistance to insulin and explored the relationships between insulin resistance and the TNF-α system (serum TNF-α, serum-soluble TNF receptors). This is the first documented case where peripheral levels of TNF-α system activity (TNF-α, soluble TNF receptors) are not a major factor responsible for the evolution of insulin resistance, at least not in nonobese Japanese type 2 diabetic patients.

2. Subjects and methods

Eighty-eight nonobese Japanese type 2 diabetic patients who visited Kansai-Denryoku Hospital were enrolled for the present study. Type 2 diabetes mellitus was diagnosed based on the World Health Organization criteria [14]. The patients showed no evidence of acute infectious illness at the time of the study. The duration of diabetes was 11.0 ± 0.8 years (mean ± SEM) (range, 1-35 years). Seventy-six of 88 diabetic patients were taking sulfonylureas (gliclazide), and the rest were treated on a dietary regimen with no medication to alter blood glucose level. No patients have received insulin therapy. All subjects had ingested at least 150 g of carbohydrate for the 3 days preceding the study. None of the subjects had significant renal, hepatic, or cardiovascular disease. Patients did not consume alcohol or perform heavy exercise for at least 1 week before the study. Blood pressure was also measured.

Blood was drawn in the morning after a 12-hour fast. Plasma glucose was measured with glucose oxidase method. The triglycerides, total cholesterol, and high-density lipoprotein cholesterol (HDL-C) were also measured. Serum insulin was measured using a 2-site immunoradiometric assay (Insulin Riabead II, Dainabot, Osaka, Japan). Coefficients of variation were 4% for insulin greater than 25 μU/mL and 7% for insulin less than 25 μU/mL. Serum leptin and adiponectin concentrations were measured with a radioimmunoassay kit (Linco Research, St Charles, Mo) as described previously [7,8]. The intra-assay and interassay coefficients of variation were less than 5% for leptin and adiponectin. Serum TNF-α concentrations were measured by enzyme immunoassay kit (Quantikine HS Human TNF-α immunoassay kit, R&D Systems, Inc, Minneapolis, Minn), and serum concentrations of sTNF-R1 and sTNF-R2 were measured by enzyme-linked immunosorbent assay (BIOTRAK, Amersham Life Sciences, Uppsala, Sweden), as described previously [15]. The limits of sensitivity for TNF-α, sTNF-R1, and sTNF-R2 were 0.5, 25, and 50 pg/mL, respectively. Samples for insulin, leptin, adiponectin, and TNF-α system (TNF-α, sTNF-R1, and sTNF-R2) were prepared, frozen, and stored at −70°C until the assay.

The estimate of insulin resistance by homeostasis model assessment (HOMA-IR) was calculated with the following formula: fasting serum insulin (μU/mL) × fasting plasma glucose (mmol/L)/22.5 [16]. The HOMA-IR value of normal tolerant subjects was 1.6 ± 0.9 (mean ± SD), and we defined the values greater than 2.5 as an insulin-resistant state and the values less than 2.5 as an insulin-sensitive state [2,5,17]. The threshold value (2.5) for insulin resistance in our study is similar to that (2.77) in nonobese subjects with no metabolic disorders reported by Bonora et al [18]. It may be argued that the use of sulfonylureas in patients with diabetes might significantly affect the estimate of insulin resistance by HOMA, as these drugs are known to decrease fasting plasma glucose without substantially changing fasting plasma insulin [19]. It seems, however, unlikely because Bonora et al [20] and Emoto et al [21] showed that in the validation studies of HOMA, the correlation of insulin sensitivity measured by such method and that measured by the glucose clamp was not substantially different in diet-treated and sulfonylurea-treated type 2 diabetes. Another problem is that pancreatic B-cell function per se might affect HOMA-IR in Japanese type 2 diabetic patients because these patients are accompanied by mild impairments in pancreatic B-cell function [2]. In our present study, however, fasting C-peptide level was greater than 0.8 ng/mL, indicating that their pancreatic function is not severely impaired. Therefore, we used HOMA-IR in diet-treated and sulfonylurea-treated diabetic patients, taking into account pancreatic insulin secretion.

Table 1
Clinical characteristics in insulin-resistant and insulin-sensitive diabetic patients

	Insulin-resistant	Insulin-sensitive	*P*
No. of subjects	32	56	
Age (y)	61.9 ± 1.7	63.2 ± 1.1	.252
Men/women	25/7	38/18	.155
HOMA-IR	3.58 ± 0.22	1.58 ± 0.07	<.001
Diabetes duration (y)	10.7 ± 1.5	11.2 ± 0.8	.376
Smoking (no/yes)	25/7	42/14	.307
SU/diet	27/5	49/7	.343
BMI (kg/m^2)	23.7 ± 0.3	22.4 ± 0.3	.003
HbA1c (%)	7.4 ± 0.2	6.8 ± 0.1	.007
Triglycerides (mg/dL)	153 ± 12	104 ± 5	<.001
Total cholesterol (mg/dL)	214 ± 6	198 ± 5	.026
Leptin (ng/mL)	6.4 ± 0.8	4.7 ± 0.4	.018
HDL-C (mg/dL)	54 ± 2	61 ± 2	.012
Adiponectin (μg/mL)	10.7 ± 1.1	16.9 ± 1.6	.005
Fasting glucose (mg/dL)	150 ± 4	135 ± 3	.003
Fasting insulin (μU/mL)	9.8 ± 0.6	4.7 ± 0.2	<.001
Systolic blood pressure (mm Hg)	139 ± 3	135 ± 3	.107
Diastolic blood pressure (mm Hg)	86 ± 2	79 ± 1	.001
TNF-α (pg/mL)	3.70 ± 0.49	3.15 ± 0.19	.107
sTNF-R1 (pg/mL)	1132 ± 55	1208 ± 55	.185
sTNF-R2 (pg/mL)	2025 ± 88	2073 ± 67	.333

3. Statistical analysis

Data are presented as mean values ± SEM. Statistical analyses were conducted using the StatView 5 system (Statview, Berkeley, Calif). The mean values of the 2 groups were compared with Student t test. Spearman rank correlation coefficient analysis was also performed to calculate a correlation. $P < .05$ was considered as significant.

4. Results

The subjects studied were all Japanese type 2 diabetic patients (63 men and 25 women) with an age range of 43 to 84 years (62.8 ± 1.0 years) and a BMI of 17.1 to 26.7 kg/m^2 (21.0 ± 0.8 kg/m^2). They were all nonobese [22]. The fasting plasma glucose was 141 ± 3 mg/dL, and glycosylated hemoglobin (HbA1c) was 7.0% ± 0.1%. Fasting insulin level was 6.56 ± 0.39 μU/mL. Serum triglycerides, total cholesterol levels, and HDL-C levels were 121 ± 6, 204 ± 4, and 59 ± 2 mg/dL, respectively. Serum leptin and adiponectin concentrations were 5.3 ± 0.4 ng/mL and 14.6 ± 1.2 pg/mL, respectively. There was a wide variation in insulin resistance calculated by HOMA in our diabetic patients (range, 0.51-7.17; mean ± SD, 2.30 ± 0.15). Thirty-two (36%) of 88 patients had HOMA-IR greater than 2.5, indicating that they are insulin-resistant [4,5]. On the other hand, serum TNF-α, soluble TNF-R1(sTNF-R1), and soluble TNF-R2 (sTNF-R2) were 3.35 ± 0.22 (range, 1.6-15.7), 1180 ± 43 (range, 699-2920), and 2055 ± 56 pg/mL (range, 1250-3860 pg/mL), respectively.

Table 1 shows the clinical profile between insulin-resistant and insulin-sensitive type 2 diabetic patients. Compared with insulin-sensitive type 2 diabetic patients, insulin-resistant patients had significantly higher levels of BMI, HbA1c, triglycerides, total cholesterol, leptin, and diastolic blood pressure and lower concentrations of HDL-C and adiponectin. No significant difference was observed in age, sex, duration of diabetes, smoking, systolic blood pressure, and the 3 measures of TNF-α system (TNF-α, sTNF-R1, and sTNF-R2) between the 2 groups.

Table 2
Correlation of TNF-α, sTNF-R1, and sTNF-R2 to measures of variables in diabetic patients

	TNF-α		sTNF-R1		sTNF-R2	
	r	P	r	P	r	P
BMI	−0.062	.563	−0.013	.904	−0.159	.137
Systolic blood pressure	0.042	.712	0.208	.682	0.154	.177
Diastolic blood pressure	0.136	.233	0.006	.956	−0.009	.940
HbA1c	0.028	.790	−0.031	.769	−0.128	.233
Fasting glucose	−0.067	.948	−0.073	.496	−0.161	.133
Fasting insulin	0.048	.653	0.026	.811	−0.012	.908
HOMA-IR	0.026	.806	−0.008	.938	−0.061	.571
Triglycerides	0.082	.442	0.011	.920	−0.041	.705
Leptin	−0.204	.059	−0.004	.968	−0.093	.389
Adiponectin	−0.188	.089	0.111	.314	0.148	.180

The correlation between the 3 measures of TNF-α system (TNF-α, sTNF-R1, and sTNF-R2) and the factors associated with insulin resistance (BMI, systolic blood pressure, diastolic blood pressure, HbA1c, fasting glucose, fasting insulin, HOMA-IR, triglycerides, leptin, and adiponectin) was next investigated in our diabetic patients (Table 2). Peripheral levels of the 3 measures of the TNF-α system (TNF-α, sTNF-R1, and sTNF-R2) were not associated with these variables.

5. Discussion

Type 2 diabetes is a heterogenous syndrome characterized by insulin resistance and/or defective insulin secretion [1]. There seems to be racial difference in insulin resistance in type 2 diabetes. Haffner et al [23] surveyed the prevalence of white type 2 diabetic patients and found that 92% of type 2 diabetic patients were insulin-resistant. Chaiken et al [24] reported that 60% of type 2 diabetic patients with BMI less than 30 kg/m^2 were insulin-resistant in African-American populations. We recently demonstrated that 40% of type 2 diabetic patients are insulin-resistant in nonobese Japanese type 2 diabetic patients [4,5]. Whereas the patients with type 2 diabetes already manifest some elements of inflammation, the intriguing feature that nonobese Japanese type 2 diabetic patients are divided into 2 variants enables us to explore whether some inflammatory markers such as TNF-α participated in the worsening of insulin resistance. We therefore investigated TNF-α and sTNF-R in nonobese Japanese type 2 diabetic patients stratified into 2 different groups: one with insulin resistance and the other with normal insulin sensitivity.

The reason why Japanese type 2 diabetic patients are not always associated with insulin resistance is unclear, but it may be due to the fact that mean BMI in our type 2 diabetic patients is 21.0 kg/m^2 less than that in white populations (average BMI 30 kg/m^2). Chang et al [25] recently reported that only 23.6% of Korean type 2 diabetic patients are insulin-resistant. Their mean level of BMI was 22.6 kg/m^2.

Using HOMA-IR and/or minimal model analysis, we have investigated the factors underlying insulin resistance in nonobese Japanese type 2 diabetic patients [2-9]. Whereas BMI and triglycerides are considered to be the most important factors responsible for the evolution of insulin resistance, regional abdominal adipose tissue distribution per se contributes to insulin resistance in nonobese Japanese type 2 diabetic patients [19]. In contrast to white and African-American populations, subcutaneous and visceral fat areas are independently associated with insulin resistance in nonobese Japanese type 2 diabetic patients [26,27]. Not only serum triglycerides but also serum leptin and adiponectin levels are shown to be associated with insulin resistance in our populations [4,5,7,8]. Serum triglycerides level is positively correlated to visceral fat area [9]. Serum leptin level is positively correlated to subcutaneous fat areas, whereas serum adiponectin level is negatively correlated to

visceral fat areas [7,8]. Furthermore, we recently demonstrated that inflammation per se is independently associated with insulin resistance in nonobese Japanese type 2 diabetic patients [6]. We subsequently found that C-reactive protein, 1 of the inflammatory markers, is not only associated with insulin resistance but also with BMI and adipocytokine such as leptin and adiponectin (data not shown). Thus, the factors underlying insulin resistance in nonobese Japanese type 2 diabetic patients are hypothesized to be linked to adipose tissue–related insulin resistance.

Another candidate that is associated with adipose tissue–related insulin resistance is TNF-α, a potent proinflammatory cytokine [10]. Hotamisligil and Spiegelman [28] were the first workers who proposed that TNF-α represents a key mediator of obesity-linked insulin resistance. Overexpression of TNF-α from adipose tissue is shown in different rodent models of obesity. Dandona et al [12] showed that plasma concentration of TNF-α is increased among obese subjects, and it decreases with weight loss. In vitro studies have shown that TNF-α inhibits insulin-stimulated glucose uptake in adipocytes in vitro by decreasing phosphorylation of the insulin receptor [29].

In the present study, we used serum TNF-α, soluble TNF-R1, and soluble TNF-R2 as an index of TNF-α system activity since peripheral levels of TNF receptor remain elevated for a longer time than TNF-α itself and reflect the degree of TNF-α activation more accurately than the measurement of TNF-α itself. Using the 3 measures of TNF-α system activity, we first demonstrated that TNF-α system activity is not responsible for insulin resistance, at least not in nonobese Japanese type 2 diabetic patients. This is a surprising finding because TNF-α is suggested to have a key role in the assessment of insulin resistance of obese and type 2 diabetic patients [10,28]. Thus, the reason why we could not find the relationship between insulin resistance and peripheral levels of TNF-α system in our patients is not known, but it may be due to the difference in clinical characteristics studied. The previous studies supporting the relationship between insulin resistance and TNF-α are derived from the studies dealing with the obese diabetic patients [10-13]. Obese subjects are shown to have higher concentration of TNF-α than nonobese subjects. Moreover, adipose tissue TNF-R2 messenger RNA is shown to be correlated with BMI and hyperinsulinemia in obese diabetic patients. Weight loss is accompanied by a decrease in serum TNF-α concentration and an increase in insulin sensitivity.

On the other hand, there is some literature supporting our present finding that peripheral levels of TNF-α system activity are not associated with insulin resistance in human subjects. Kellerer et al [30] found no correlation between plasma TNF-α and insulin resistance in the offspring of type 2 diabetic patients. Two investigators [31,32] have shown that administration of antibodies or antagonists to TNF-α have not improved insulin sensitivity in insulin-resistant individuals. Zavarotoni et al [33] recently demonstrated that differences in TNF-α activity do not appear to contribute to the marked variation in insulin action that occurs in healthy individuals. Ghanim et al [34] very recently showed that TNF-α is not related to HOMA-IR in obese subjects. Thus, it may be speculated that adipose tissue–linked TNF-α system activity might function locally at the level of the adipocyte in a paracrine or autocrine fashion in our study's diabetic patients. Alternatively, adipose tissue may not play a major role in the determination of peripheral levels of TNF-α system activity in our nonobese, well-controlled, unique Japanese type 2 diabetic patients.

In summary, we demonstrated for the first time that although the number of patients with type 2 diabetes is limited, peripheral levels of TNF-α system activity do not appear to be a major explanation of the mechanisms underlying insulin resistance at least in nonobese well-controlled Japanese type 2 diabetic patients. This idea can be inferred from our present study that peripheral levels of TNF-α system activity are not associated with serum leptin and adiponectin which are another index of insulin resistance in human beings [10].

Acknowledgment

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References

[1] Gerich JE. The genetic basis of type 2 diabetes mellitus: impaired insulin secretion versus impaired insulin sensitivity (Review). Endocr Rev 1998;19:491-503.

[2] Taniguchi A, Nakai Y, Fukushima M, et al. Pathogenic factors responsible for glucose tolerance in patients with NIDDM. Diabetes 1992;41:1540-6.

[3] Nagasaka S, Tokuyama K, Kusaka I, et al. Endogenous glucose production and glucose effectiveness in type 2 diabetic subjects derived from stable-labeled minimal model approach. Diabetes 1999;48: 1054-60.

[4] Taniguchi A, Fukushima M, Sakai M, et al. The role of the body mass index and triglyceride levels in identifying insulin-sensitive and insulin-resistant variants in Japanese non–insulin-dependent diabetic patients. Metabolism 2000;49:1001-5.

[5] Taniguchi A, Fukushima M, Sakai M, et al. Remnant-like particle cholesterol, triglycerides, and insulin resistance in nonobese Japanese type 2 diabetic patients. Diabetes Care 2000;23:1766-9.

[6] Taniguchi A, Nagasaka S, Fukushima M, et al. C-reactive protein and insulin resistance in non-obese Japanese type 2 diabetic patients. Metabolism 2002;51:1578-81.

[7] Okumura T, Taniguchi A, Nagasaka S, et al. Relationship of regional adiposity to serum leptin level in nonobese Japanese type 2 diabetic male patients. Diabetes Metab 2003;29:15-8.

[8] Yatagai T, Nagasaka S, Fukushima M, et al. Hypoadiponectinemia is associated with visceral fat accumulation and insulin resistance in subjects with type 2 diabetes mellitus. Metabolism 2003;52:1274-8.

[9] Taniguchi A, Nakai Y, Sakai M, et al. Relationship of regional adiposity to insulin resistance and serum triglyceride levels in nonobese Japanese type 2 diabetic patients. Metabolism 2002;51:544-8.

[10] Pittas AG, Joseph NA, Greenberg AS. Adipocytokines and insulin resistance. J Clin Endocrinol Metab 2004;89:447-52.
[11] Hotamisligil GH, Arner P, Caro JF, et al. Increased adipose tissue expression of tumor necrosis factor-α in human obesity and insulin resistance. J Clin Invest 1995;95:2409-15.
[12] Dandona P, Weinstock R, Thuse A, et al. Tumor necrosis factor-α in sera of obese patients: fall with weight loss. J Clin Endocrinol Metab 1998;83:2907-10.
[13] Katsuki A, Sumida Y, Murashima S, et al. Serum levels of tumor necrosis factor-α are increased in obese patients with non-insulin-dependent diabetes mellitus. J Clin Endocrinol Metab 1998;83:859-62.
[14] World Health Organization. Diabetes mellitus: report of a WHO study group. Geneva: World Health Org; 1985 [Tech. Rep. Ser., no. 727].
[15] Nakai Y, Hamagaki S, Takagi R, et al. Plasma concentrations of tumor necrosis factor-α (TNF-α) and soluble TNF receptors in patients with bulimia nervosa. Clin Endocrinol 2000;53:383-8.
[16] Matthews DR, Hosker JP, Rudenski AS, et al. Homeostasis model assessment: insulin resistance and B-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28: 412-9.
[17] Taniguchi A, Nakai Y, Doi K, Fukushima M, et al. Glucose effectiveness in two subtypes within impaired glucose tolerance: a minimal model analysis. Diabetes 1994;43:1211-7.
[18] Bonora E, Kiechl S, Willeit J, Oberhollenzer F, et al. Prevalence of insulin resistance in metabolic disorders: the Bruneck study. Diabetes 1998;47:1643-9.
[19] Groop LC. Drug treatment of non-insulin-dependent diabetes mellitus. In: Pickup J, Williams G, editors. Textbook of diabetes, vol. 38. Oxford (UK): Blackwell; 1997. p. 1-18.
[20] Bonora E, Targher G, Alberiche M, et al. Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity. Diabetes Care 2000;23:57-63.
[21] Emoto M, Nishizawa Y, Maekawa K, et al. Homeostasis model assessment as a clinical index of insulin resistance in type 2 diabetic patients treated with sulfonylureas. Diabetes Care 1999;22:818-22.
[22] Taniguchi A, Nakai Y, Doi K, et al. Insulin sensitivity, insulin secretion, and glucose effectiveness in obese subjects: a minimal model analysis. Metabolism 1995;44:1397-400.
[23] Haffner SM, D'Agostino Jr R, Mykkanen L, et al. Insulin sensitivity in subjects with type 2 diabetes: relationship to cardiovascular risk factors: the Insulin Resistance Atherosclerosis Study. Diabetes Care 1999;22:562-8.
[24] Chaiken RL, Banerji MA, Pasmantier RM, et al. Patterns of glucose and lipid abnormalities in black NIDDM subject. Diabetes Care 1991;14: 1036-42.
[25] Chang SA, Kim HS, Yoon KH, et al. Body mass index is the most important determining factor for the degree of insulin resistance in non-obese type 2 diabetic patients in Korea. Metabolism 2004;53: 142-6.
[26] Abate N, Garg A, Peshock RM, et al. Relationship of generalized and regional adiposity to insulin sensitivity in men with NIDDM. Diabetes 1996;45:1684-93.
[27] Banerji MA, Chaiken RL, Gordon D, et al. Does intra-abdominal adipose tissue in black men determine whether NIDDM is insulin-resistant or insulin-sensitive? Diabetes 1995;44:141-6.
[28] Hotamisligil GS, Spiegelman BM. Tumor necrosis factorα: a key component of the obesity-diabetes link. Diabetes 1994;43:1271-8.
[29] Liu LS, Spelleken M, Rohrig K, et al. Tumor necrosis factor-α acutely inhibits insulin signaling in human adipocytes. Implication of the p80 tumor necrosis factor receptor. Diabetes 1998;47:515-22.
[30] Kellerer M, Rett K, Renn W, et al. Circulating TNF-alpha and leptin levels in offsprings of NIDDM patients do not correlate to individual insulin sensitivity. Horm Metab Res 1997;29:111-4.
[31] Ofei F, Hurel S, Newkirk J, et al. Effects of an engineered human anti-TBF0-alpha antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM. Diabetes 1996;45:881-5.
[32] Paquot N, Castillo MJ, Lefebvee PJ, et al. No increased insulin sensitivity after a single administration of a recombinant human tumor necrosis factor receptor: Fc fusion protein in obese insulin resistant patients. J Clin Enodocrinol Metab 2000;85:1316-9.
[33] Zavarotoni I, Numeroso F, Dongiovanni P, et al. What is the contribution of differences in three measures of tumor necrosis factor-alpha activity to insulin resistance in healthy volunteers? Metabolism 2003; 52:1593-6.
[34] Ghanim H, Aljada A, Hofmeyer D, et al. Circulating mononuclear cells in the obese are in a proinflammatory state. Circulation 2004;110: 1564-71.

Distinct Effects of Glucose-Dependent Insulinotropic Polypeptide and Glucagon-Like Peptide-1 on Insulin Secretion and Gut Motility

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Glucose-induced insulin secretion from pancreatic β-cells depends critically on ATP-sensitive K^+ channel (K_{ATP} channel) activity, but it is not known whether K_{ATP} channels are involved in the potentiation of insulin secretion by glucose-dependent insulinotropic polypeptide (GIP). In mice lacking K_{ATP} channels ($Kir6.2^{-/-}$ mice), we found that pretreatment with GIP in vivo failed to blunt the rise in blood glucose levels after oral glucose load. In $Kir6.2^{-/-}$ mice, potentiation of insulin secretion by GIP in vivo was markedly attenuated, indicating that K_{ATP} channels are essential in the insulinotropic effect of GIP. In contrast, pretreatment with glucagon-like peptide-1 (GLP-1) in $Kir6.2^{-/-}$ mice potentiated insulin secretion and blunted the rise in blood glucose levels. We also found that GLP-1 inhibited gut motility whereas GIP did not. Perfusion experiments of $Kir6.2^{-/-}$ mice revealed severely impaired potentiation of insulin secretion by 1 nmol/l GIP and substantial potentiation by 1 nmol/l GLP-1. Although both GIP and GLP-1 increase the intracellular cAMP concentration and potentiate insulin secretion, these results demonstrate that the GLP-1 and GIP signaling pathways involve the K_{ATP} channel differently. *Diabetes* 54: 1056–1063, 2005

Oral glucose load elicits larger insulin secretion and less increase in blood glucose levels than intravenous administration of the equivalent amount of glucose (1,2). This phenomenon is mostly due to incretins, gut-derived factors including glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (3,4). GIP and GLP-1 are released from gastrointestinal endocrine K-cells and L-cells, respectively, into the blood stream in response to the ingestion of nutrients (5), which potentiates insulin secretion from pancreatic β-cells (6–9). GIP and GLP-1 exert their insulinotropic effects by binding to GIP receptors (10) and GLP-1 receptors on the β-cell surface (11), respectively, activating adenylyl cyclase (12,13), which leads to the rise in intracellular cAMP concentration that potentiates insulin secretion by activating protein kinase A– and/or cAMP–guanine nucleotide exchange factor (GEF)2–mediated signaling in normal pancreatic β-cells (14,15). Thus, GIP and GLP-1 share in part a common pathway of insulin secretion enhancement. However, many clinical findings suggest different mechanisms of GIP and GLP-1 action. In patients with type 2 diabetes, for example, the insulinotropic action of GLP-1 is well preserved whereas that of GIP is markedly reduced (16). The mechanism of the differing effects GLP-1 and GIP remains unknown.

Recent studies of GIP-receptor knockout ($GIPR^{-/-}$) mice have shown that potentiation of insulin secretion by GIP plays an important role in glucose metabolism (17). $GIPR^{-/-}$ mice have higher glucose levels in response to oral glucose load than in response to intraperitoneal load, showing that endogenous GIP plays an important role in preventing a rise in blood glucose levels after oral load. Unlike other secretagogues that stimulate insulin secretion, GIP exerts a potentiating effect on insulin secretion only in the presence of glucose (7,18,19). The glucose dependency of the insulinotropic action of GIP has been confirmed using stepwise glucose clamp in normal human subjects (9,20,21).

ATP-sensitive K^+ channel (K_{ATP} channel) null ($Kir6.2^{-/-}$ and $SUR1^{-/-}$) mice do not exhibit significant insulin secretion in response to oral glucose load (22–24). This raises the possibility that $Kir6.2^{-/-}$ mice have either a defect in glucose-induced GIP secretion from K-cells or a defect in potentiation by GIP of insulin secretion from β-cells. Because glucose-induced GIP secretion from K-cells has been shown to occur in a K_{ATP} channel–independent manner, we investigated the potentiating effect of GIP on insulin secretion from β-cells in $Kir6.2^{-/-}$ mice. We also examined the effects of GLP-1, the other important incretin hormone, on the potentiation of insulin secretion and

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AUC, area under the curve; GEF, guanine nucleotide exchange factor; GIP, glucose-dependent insulinotropic polypeptide; $GIPR^{-/-}$, GIP-receptor knockout; GLP-1, glucagon-like peptide-1; K_{ATP} channel, ATP-sensitive K^+ channel; KRBH, Krebs-Ringer bicarbonate HEPES; OGTT, oral glucose tolerance test; PKA, protein kinase A; PreTx, GIP pretreatment.

blood glucose levels after an oral glucose load in Kir6.2$^{-/-}$ mice.

RESEARCH DESIGN AND METHODS

Kir6.2$^{-/-}$ mice were generated as previously described (22). Because the Kir6.2$^{-/-}$ mice had been backcrossed to the C57BL/6 mouse strain over five generations, C57BL/6 mice were used as wild-type (Kir6.2$^{+/+}$) mice. All animal experiments were performed in accordance with the guidelines of the Animal Care Committee of Chiba and Kobe University.

GIP secretion assay in vivo. The secretion of GIP in response to oral glucose was examined in conscious male mice (18–20 weeks old, weighing 20–25 g) in vivo. After an overnight fast (16 h), Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice were administered D-glucose (150 mg/mouse in 0.5 ml) via gavage. A blood sample (~500 μl of whole blood) was taken 15 min after glucose load and separated by centrifugation at 12,000*g* for 15 min at 4°C and stored at −80°C until hormone radioimmunoassay. Blood samples for basal GIP and glucose level was taken independently 1 week before (n = 6 for both genotypes) and after (n = 6 for both genotypes) the glucose loading test. GIP concentrations and glucose levels were determined as previously described (25–27).

Oral glucose tolerance test and measurement of blood glucose and serum insulin levels. One-hundred micrograms of human GIP (in 0.1 ml), human GLP-1 (in 0.1 ml), or saline (0.1 ml) was given subcutaneously to overnight (16 h)-fasted male mice. Glucose (1.5 g/kg) was administered 5 min after GIP or GLP-1 pretreatment as a 15% solution via gavage. Blood glucose levels at 0, 10, 30, 60, 90, 120, and 180 min and serum insulin levels at 0, 10, and 30 min after the glucose load were measured as previously described (27). The areas under the curve (AUCs) were assessed for blood glucose levels ($AUC_{glucose}$) with the trapezoidal rule of suprabasal values.

Measurement of gastrointestinal transit. To evaluate gastrointestinal motility, male mice were fasted with free access to drinking water for 48 h. On the day of the experiment, the mice received an intragastric injection of 20 μl/g test solution (25% wt/vol barium sulfate suspended in water or 50% wt/vol D-glucose solution). The mice were killed 15 min later by cervical dislocation. After dissection, the length from the pylorus to the most distal point of migration of the barium (*A*) and from the pylorus to terminal ileum (*B*) was measured. Gastrointestinal transit was expressed as percentage of *A* to *B*. To determine the effects of GIP and GLP-1 on gastrointestinal motility, mice were pretreated 5 min before test solution ingestion with 100 μg human GIP or GLP-1.

Perfusion experiments of mouse pancreata. Overnight (16 h)-fasted male mice at 16–20 weeks of age were used in perfusion experiments as previously reported (28) with slight modifications. Briefly, after anesthesia with 80 mg/kg sodium pentobarbital, the superior mesenteric and renal arteries were ligated, and the aorta was tied off just below the diaphragm. The perfusate was infused from a catheter placed in the aorta and collected from the portal vein. The perfusate was Krebs-Ringer bicarbonate HEPES (KRBH) buffer supplemented with 4.6% dextran and 0.25% BSA and gassed with 95% O_2/5% CO_2. The flow rate of the perfusate was 1 ml/min. In experiments involving GIP and GLP-1, mouse pancreata were perfused with KRBH buffer containing 2.8 or 16.7 mmol/l glucose in the presence or absence of 1 nmol/l GIP or 1 nmol/l GLP-1. In experiments involving arginine and carbachol, pancreata were perfused with KRBH buffer containing 5.5 mmol/l glucose in the presence or absence of 20 mmol/l arginine or 50 μmol/l carbachol. The perfusion protocols began with a 10-min equilibration period with the same buffer used in the initial step (i.e., from 1 to 5 min) shown in the figures. The insulin levels in the perfusate were measured by an ELISA kit (Mesacup Insulin Test) from BML (Nagoya, Japan).

Measurement of insulin secretion in response to arginine and carbachol in vivo. To analyze arginine- and carbachol-induced insulin secretion, overnight (16 h)-fasted male mice were administered 250 mg/kg L(+)-arginine intravenously or 750 μg/kg carbachol intraperitoneally as previously described by Guenifi et al. (29) and Havel et al. (30). Blood samples were taken before and 2 min after load, and blood glucose and serum insulin levels were measured.

Meal ingestion test. Glucose tolerance and insulin secretory response to mixed meal was evaluated using the enteral feeding formula Twinline, which is used clinically and which consists mainly of casein from milk protein, amino acids, maltodextrin, fat from safflower oil, and tricaprilin and contains 4.05 g/dl protein, 2.78 g/dl carbohydrate, and 2.78 g/dl fat (1 kcal/ml calorie in total). After overnight fasting (16 h), male mice were administered 20 μl/g Twinline (20 kcal/g energy and 3 g/kg carbohydrate), and blood glucose levels at 0, 30, 60, 120, and 180 min and serum insulin levels at 0, 30, and 60 min after the glucose load were measured.

Reagents. Synthetic human GIP and GLP-1 were purchased from Peptide Institute (Osaka, Japan). Arginine [L(+)-arginine monohydrochloride] was from Nacalai (033-23), and carbachol (carbamylcholine chloride, C-4382) was from Sigma. Twinline enteral formula was from Otsuka Pharmaceuticals (Tokushima, Japan).

Statistical calculations. All values are shown as means ± SE. *P* values were calculated with unpaired Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

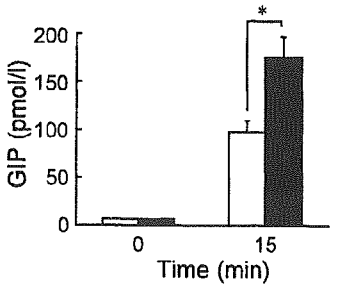

FIG. 1. GIP secretion in response to oral glucose. Plasma GIP levels in Kir6.2$^{+/+}$ (□; n = 12) and Kir6.2$^{-/-}$ (■; n = 12) mice. *$P < 0.0001$.

RESULTS

Glucose-induced GIP secretion. Oral glucose load elicited a significant increase in GIP secretion after 15 min in Kir6.2$^{+/+}$ mice (113.3 ± 2.6 pmol/l, n = 12) (Fig. 1). GIP secretion was significantly increased also in Kir6.2$^{-/-}$ mice (207.3 ± 14.7 pmol/l, n = 12), suggesting that glucose-induced GIP secretion is K_{ATP} channel independent. Interestingly, the increment in plasma GIP in Kir6.2$^{-/-}$ mice was enhanced in Kir6.2$^{-/-}$ mice ($P <$ 0.0001).

Glucose-lowering effect of GIP in vivo. Oral glucose tolerance test (OGTT) was performed on mice pretreated with or without GIP, as previously reported of GLP-1 (31). GIP pretreatment (PreTx) significantly increased glucose tolerance in Kir6.2$^{+/+}$ mice [$AUC_{glucose}$; PreTx(−), 2,146 ± 18 mmol/l in 180 min; PreTx(+), 1,649 ± 78 mmol/l in 180 min; $P < 0.005$] (Fig. 2*A*). However, GIP pretreatment failed to increase glucose tolerance in Kir6.2$^{-/-}$ mice [$AUC_{glucose}$; PreTx(−), 1,901 ± 75 mmol/l in 180 min; PreTx(+) 2,191 ± 375 mmol/l in 180 min; not significant] (Fig. 2*B*), indicating that the glucose-lowering effect of GIP is abolished completely in Kir6.2$^{-/-}$ mice.

Effect of GIP on gastrointestinal transit. We assessed gut motility by measuring gastrointestinal transit of orally ingested barium sulfate. GIP did not affect gastrointestinal transit [67.1 ± 6.7% in PreTx(−) and 74.9 ± 6.0% in PreTx(+)] in Kir6.2$^{+/+}$ mice, which shows that GIP does not inhibit gastrointestinal transit, at least in our protocol (Fig. 2*C*). However, there was a significant increase in gastrointestinal transit of oral glucose load. To evaluate involvement of the K_{ATP} channels in glucose-responsive enteric neurons in regulating gut motility of glucose load, we compared transit of oral glucose load in Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice. Gastrointestinal transit was similarly increased by oral glucose in Kir6.2$^{+/+}$ [glucose (−), 41.9 ± 2.1%; glucose (+), 57.5 ± 4.0%] and Kir6.2$^{-/-}$ [glucose (−), 34.1 ± 1.7%; glucose (+), 61.4 ± 1.7%] mice (Fig. 2*D*), indicating that gut motility is not regulated by K_{ATP} channel–mediated glucose sensing in enteric neurons.

Glucose-lowering effect of GLP-1 in vivo. We then performed OGTTs with and without GLP-1 pretreatment, as was done with GIP. GLP-1 pretreatment reduced the elevation in blood glucose significantly in Kir6.2$^{+/+}$ mice

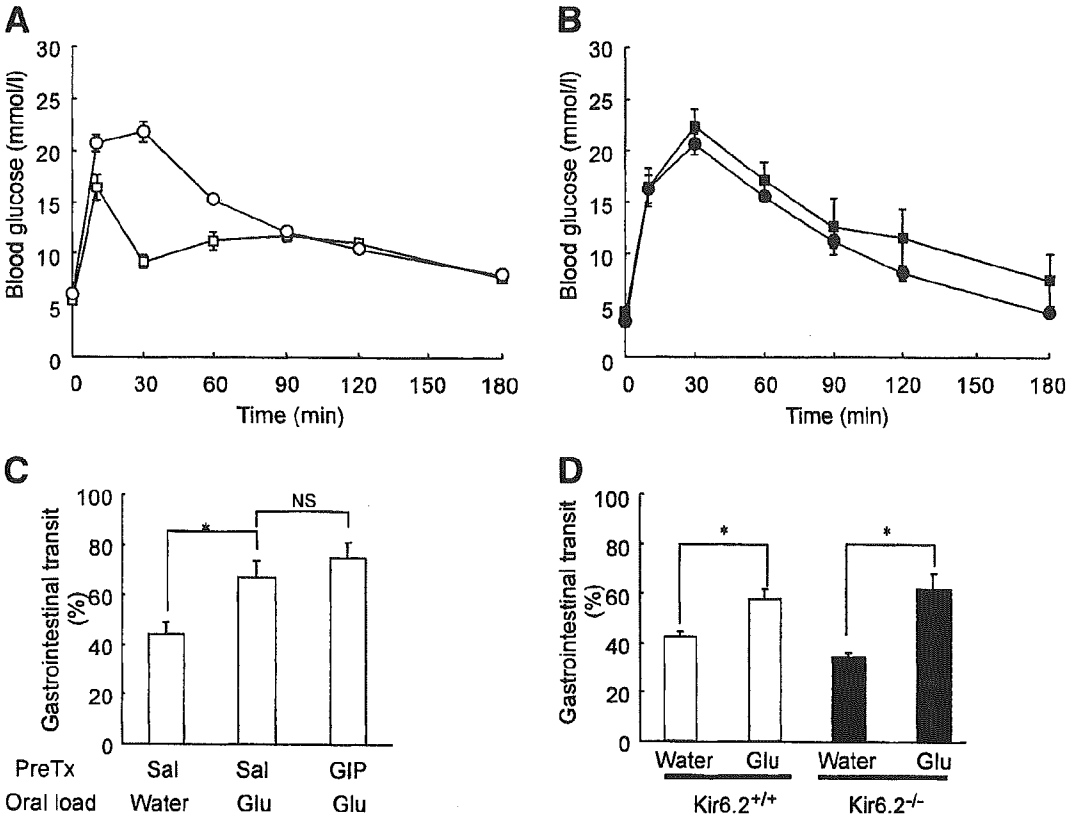

FIG. 2. Effect of GIP on blood glucose and gastrointestinal transit. *A* and *B*: Effect of GIP on blood glucose levels after oral glucose challenge in Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice. Changes in blood glucose levels of Kir6.2$^{+/+}$ (*A*) and Kir6.2$^{-/-}$ (*B*) mice (n = 5–9, for each group) during OGTT are shown. Kir6.2$^{+/+}$ (*A*) and Kir6.2$^{-/-}$ (*B*) mice were pretreated subcutaneously with saline (●) or GIP (■), and glucose was administered orally. *C*: Effect of GIP on gut motility in Kir6.2$^{+/+}$ mice. Gastrointestinal transit was measured in the three groups (n = 4 for each group). The mice were pretreated subcutaneously with saline (Sal) or GIP and then administered barium sulfate orally suspended in glucose (Glu) or water. *P < 0.05; NS, not significant. *D*: Effect of glucose on gut motility in Kir6.2$^{+/+}$ (□) and Kir6.2$^{-/-}$ (■) mice. Gastrointestinal transit was measured in Kir6.2$^{+/+}$ (n = 9) and Kir6.2$^{-/-}$ (n = 9) mice in response to orally administered barium sulfate suspended in glucose or water. *P < 0.005.

[$AUC_{glucose}$; PreTx(−), 2,057 ± 86 mmol/l in 180 min; PreTx(+), 1,347 ± 127 mmol/l in 180 min; P < 0.005] (Fig. 3*A*) as well as Kir6.2$^{-/-}$ mice [$AUC_{glucose}$; PreTx(−), 2,513 ± 156 mmol/l in 180 min; PreTx(+) 1,403 ± 155 mmol/l in 180 min; P < 0.0001] (Fig. 3*B*).

Effect of GLP-1 on gastrointestinal transit in vivo. We also examined the effect of GLP-1 on gut motility. Gastrointestinal transit was significantly suppressed by GLP-1 pretreatment (Fig. 3*C*). GLP-1 was similarly effective on gut motility in Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice, suggesting that the effect of GLP-1 is independent of K_{ATP} channel activity. In addition, GLP-1 similarly suppressed gastrointestinal transit in glucose-loaded and water-loaded mice, showing that GLP-1 and glucose regulate gut motility through independent mechanism.

Potentiation of insulin secretion by GIP and GLP-1 in vivo. We examined insulin secretion during OGTT with and without GIP or GLP-1 pretreatment (Fig. 4). Ten minutes after glucose loading, serum insulin levels were already elevated in Kir6.2$^{+/+}$ mice (77.2 ± 11.5 pmol/l at 0 min; 275.5 ± 42.5 pmol/l at 10 min) (Fig. 4). Insulin secretion at 10 min was significantly enhanced by GIP pretreatment (440.8 ± 53.8 pmol/l, P < 0.05) or by GLP-1 pretreatment (474.7 ± 49.2 pmol/l, P < 0.05). Secretion in Kir6.2$^{+/+}$ mice was no longer enhanced by GIP or GLP-1 pretreatment at 30 min, when the blood glucose levels are lower (Figs. 2*A* and 3*A*).

Interestingly, the insulinotropic effect of GIP was completely absent in Kir6.2$^{-/-}$ mice at 10 min (83.1 ± 9.0 pmol/l at 0 min; 66.2 ± 17.0 pmol/l at 10 min; not significant); however, there was significant potentiation of insulin secretion at 10 min by GLP-1 pretreatment (242.9 ± 13.0 pmol/l, P < 0.0005) (Fig. 4). In contrast, at 30 min after glucose load, there was significant potentiation of insulin secretion in 30 min in Kir6.2$^{-/-}$ mice both by GIP pretreatment (245.5 ± 8.51 pmol/l) and by GLP-1 pretreatment (240.4 ± 21.1 pmol/l), even though glucose-induced insulin secretion was not observed (112.8 ± 12.5 pmol/l) (Fig. 4).

Effects of GIP and GLP-1 on insulin secretion in perfused pancreas. To examine the time course of the insulin secretory response to GIP and GLP-1 in Kir6.2$^{-/-}$ mice, perfusion experiments were performed in the absence (Fig. 5*A*) or presence of GIP (Fig. 5*B* and *C*) or GLP-1 (Fig. 5*D*). In Kir6.2$^{+/+}$ mice, 16.7 mmol/l glucose elicited insulin secretion [the amount of secreted insulin ($AUC_{insulin}$) after glucose stimulation (from 5 to 25 min); 61.4 ± 5.5 ng in 20 min, n = 3] (Fig. 5*A*), which was further potentiated by 1 nmol/l [$AUC_{insulin}$; 217.9 ± 12.3 ng, n = 3, P < 0.005 vs. GIP(−)] or 10 nmol/l GIP [$AUC_{insulin}$; 278.8 ± 25.4 ng, n = 3, P < 0.05 vs. GIP(−)] (Fig. 5*B* and *C*). In contrast, in Kir6.2$^{-/-}$ mice, 16.7 mmol/l glucose barely elicited a rise in insulin secretion ($AUC_{insulin}$; 23.3 ± 2.7 ng, n = 3) (Fig. 5*A*), and there was only slight potentiation in insulin secretion by 1 nmol/l ($AUC_{insulin}$; 37.1 ± 4.2 ng, n =

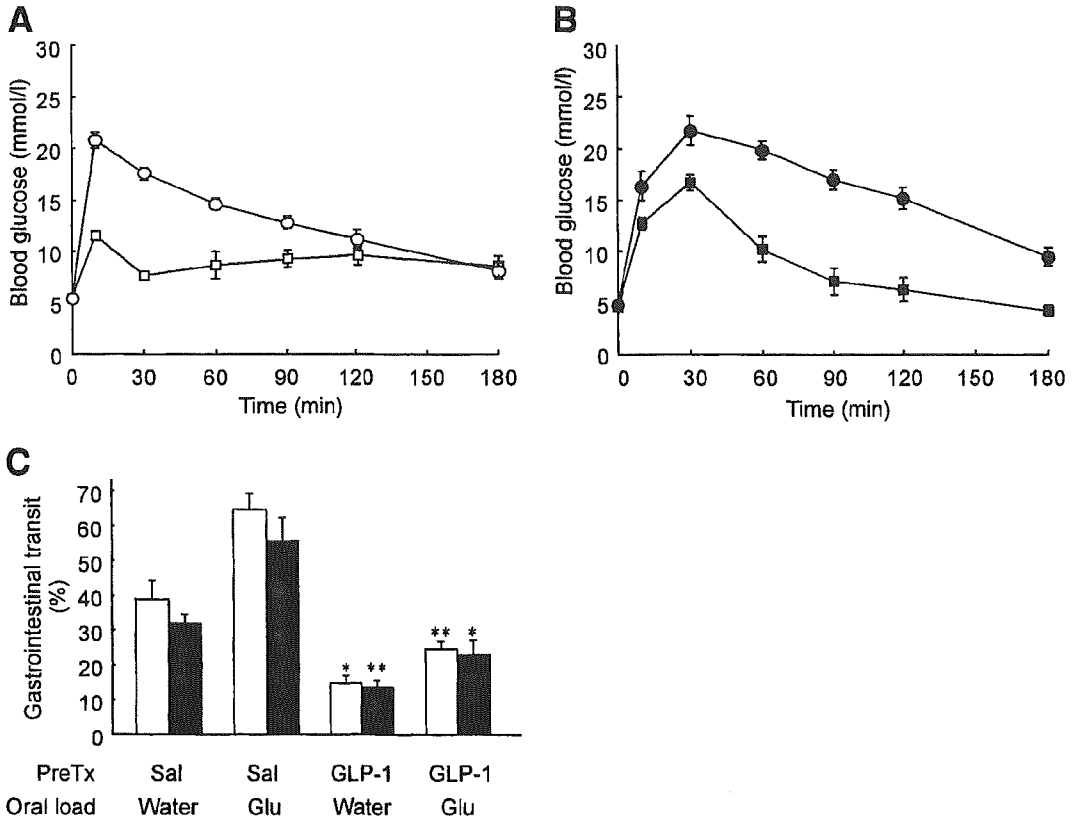

FIG. 3. Effect of GLP-1 on blood glucose after oral glucose challenge and gastrointestinal transit in Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice. *A* and *B*: Changes in blood glucose levels in Kir6.2$^{+/+}$ (*A*) and Kir6.2$^{-/-}$ (*B*) mice (n = 10–14, for each group) during OGTT are shown. Mice were pretreated subcutaneously with saline (●) or GLP-1 (■) or and were administered orally ingested glucose. *C*: Effect of glucose on gut motility in Kir6.2$^{+/+}$ (□) and Kir6.2$^{-/-}$ (■) mice. Gastrointestinal transit was measured (n = 7–9 for each group) as in Fig. 2*C*. The mice were pretreated subcutaneously with saline (Sal) or GLP-1 and then administered barium sulfate orally suspended in glucose (Glu) or water. *P < 0.005 and **P < 0.0001 for comparison between GLP-1–pretreated (GLP-1) versus GLP-1–untreated (Sal) mice.

3) or 10 nmol/l GIP ($AUC_{insulin}$; 55.8 ± 12.7 ng, n = 3) (Fig. 5*B* and *C*). The potentiation of insulin secretion by 1 nmol/l GLP-1 also was attenuated in Kir6.2$^{-/-}$ mice ($AUC_{insulin}$; 103.8 ± 40.6 ng, n = 3) compared with that of Kir6.2$^{+/+}$ mice ($AUC_{insulin}$; 329.1 ± 20.1 ng, n = 3), but the secretion was nevertheless more potent than that by 1 nmol/l GIP (Fig. 5*D*). When insulin secretion was assessed

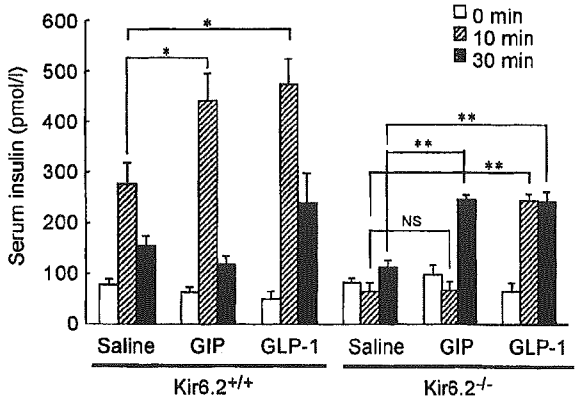

FIG. 4. Effect of GIP and GLP-1 pretreatment on insulin secretion after oral glucose challenge. Serum insulin levels in Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice before (□, n = 5–9) and 10 min (▨, n = 5–9) or 30 min (■, n = 5–9) after glucose load. *P < 0.05; **P < 0.0005.

by the $AUC_{insulin}$, 1, 10, and 1 nmol/l GLP-1 potentiated insulin secretion in Kir6.2$^{+/+}$ mice 3.5-, 3.5-, and 5.4-fold, respectively (Fig. 5*E*). In contrast, in Kir6.2$^{-/-}$ mice, 1 and 10 nmol/l GIP increased insulin secretion only by 1.6- and 2.4-fold, whereas 1 nmol/l GLP-1 increased insulin secretion by 4.5-fold (Fig. 5*E*). In addition, glucose-induced insulin secretion in Kir6.2$^{-/-}$ mice became apparent in the presence of 1 nmol/l GLP-1 [fold increase in the insulin secretory rate before and after stimulation with 16.7 mmol/l glucose; 1.52 ± 0.10-fold in the absence of GLP-1 (n = 3) (Fig. 5*A*), 4.14 ± 0.06-fold in 1 nmol/l GLP-1 (n = 3) (Fig. 5*D*); P < 0.05], indicating that Kir6.2$^{-/-}$ mice were endued with glucose responsiveness by stimulation with 1 nmol/l GLP-1.

Insulin secretory response to arginine, cholinergic stimuli, and mixed meal. Insulin secretion by arginine in vivo was significantly impaired in Kir6.2$^{-/-}$ mice (Fig. 6*A*), but marked secretion was observed 2 min after administration of carbachol in vivo in both Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice (Fig. 6*B*). The insulin secretory response was also examined in perfusion experiments. Similar to the findings in vivo, insulin secretion from Kir6.2$^{-/-}$ pancreata was markedly impaired in response to 20 mmol/l arginine [$AUC_{insulin}$ during stimulation (from 5 to 10 min); 39.1 ± 4.2 ng in Kir6.2$^{+/+}$ mice (n = 3), 8.4 ± 2.8 ng in Kir6.2$^{-/-}$ mice (n = 3)] but remained unaffected in response to 50

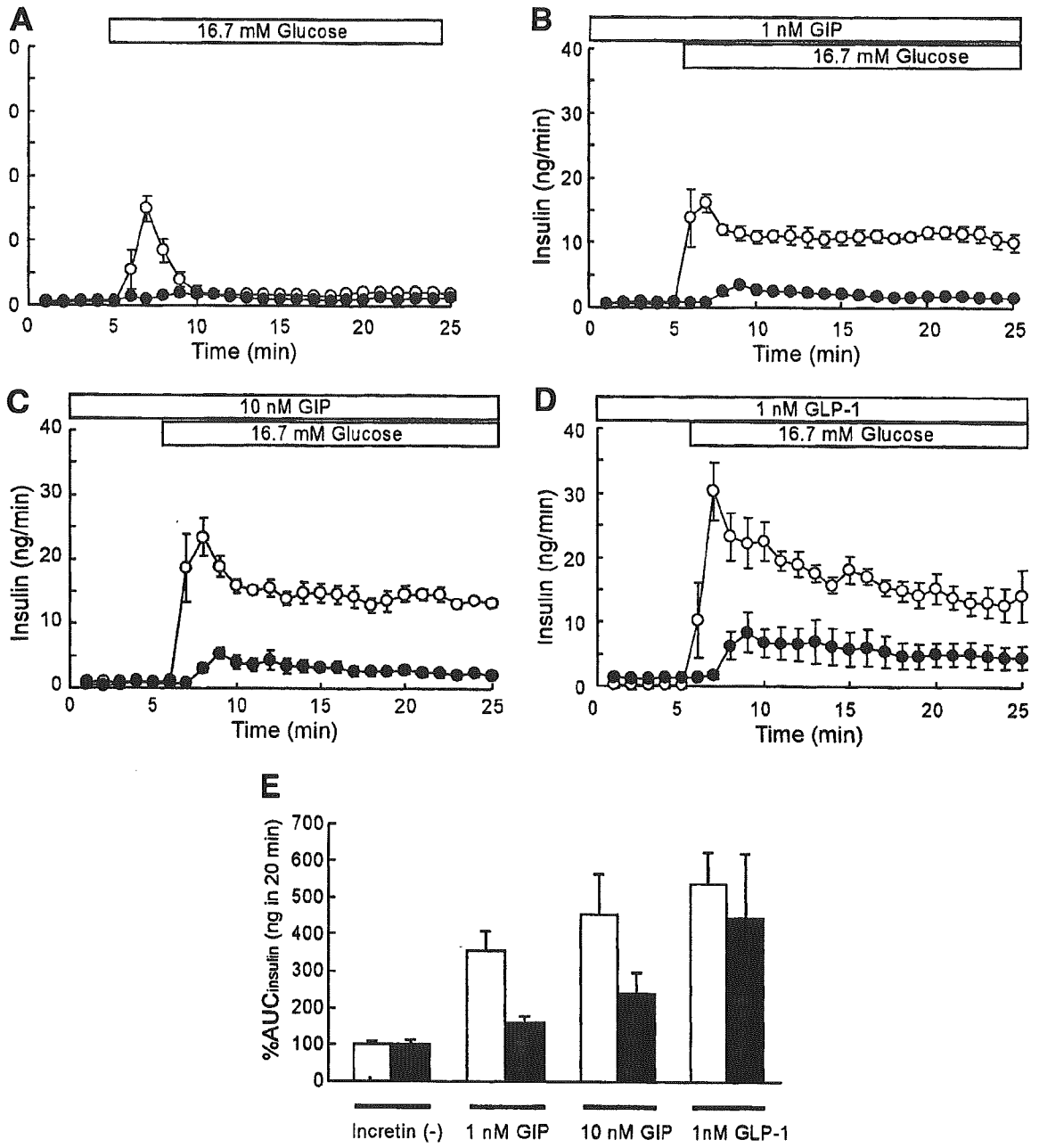

FIG. 5. Insulin secretion in perfused pancreata in response to high glucose, GIP, and GLP-1. *A–D*: Insulin secretion from perfused pancreata of Kir6.2$^{+/+}$ (○) and Kir6.2$^{-/-}$ (●) mice is shown. Glucose concentration was shifted from 2.8 to 16.7 mmol/l at 5 min in the absence of incretin (*A*) or in the presence of 1 nmol/l GIP (*B*), 10 nmol/l GIP (*C*), or 1 nmol/l GLP-1 (*D*). The data represent the mean ± SE of three mice. *E*: The amounts of secreted insulin of Kir6.2$^{+/+}$ (□) and Kir6.2$^{-/-}$ (■) mice after glucose stimulation are expressed as the $AUC_{insulin}$ from 5 to 25 min in *A–D*. Data are percent relative to that in the absence of incretin.

μmol/l carbachol [$AUC_{insulin}$ during stimulation (from 20 to 25 min); 8.7 ± 1.5 ng in Kir6.2$^{+/+}$ mice (n = 3), 10.6 ± 1.2 ng in Kir6.2$^{-/-}$ mice (n = 3)] (Fig. 6*C*). We also compared glucose tolerance and insulin secretion in response to orally ingested meal in Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice (Fig. 6*D* and *E*). In Kir6.2$^{-/-}$ mice, glucose tolerance was significantly impaired in response to mixed meal (Fig. 6*D*), and early-phase insulin secretion was significantly diminished (serum insulin levels 30 min after meal ingestion; Kir6.2$^{+/+}$, 322.2 ± 47.5 pmol/l; Kir6.2$^{-/-}$, 134.0 ± 14.9 pmol/l, $P < 0.005$) (Fig. 6*E*).

DISCUSSION

GIP is released from gastrointestinal endocrine K-cells (32) in a glucose-dependent manner (7,33). Although glucose-induced insulin secretion from pancreatic β-cells is critically dependent on K_{ATP} channel function (22–24), our present study indicated that glucose-induced GIP secretion occurs independently of K_{ATP} channel function (Fig. 1).

Because GIP pretreatment did not reduce the elevation of blood glucose in Kir6.2$^{-/-}$ mice after oral glucose load

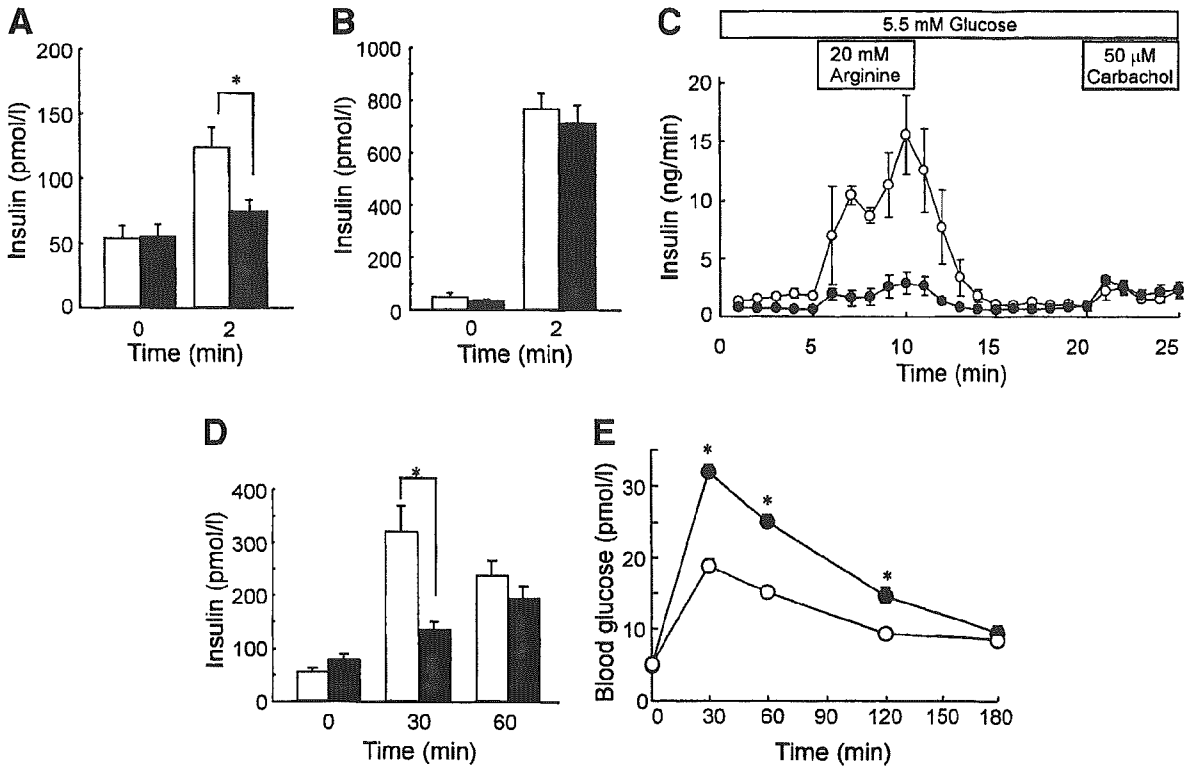

FIG. 6. Insulin secretion and glucose tolerance in response to arginine, carbachol, and a mixed meal. *A*: Early phase of insulin secretion in response to arginine is shown. Serum insulin levels in Kir6.2$^{+/+}$ (□, n = 8) and Kir6.2$^{-/-}$ (■, n = 9) mice 2 min after intravenous arginine administration. *P < 0.005. *B*: The insulin secretion in response to carbachol is shown. Serum insulin levels in Kir6.2$^{+/+}$ (□, n = 9) and Kir6.2$^{-/-}$ (■, n = 10) mice 2 min after intraperitoneal carbachol administration. There is no significant difference in serum insulin levels between Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice. *C*: Insulin secretion from perfused pancreata of Kir6.2$^{+/+}$ (○) and Kir6.2$^{-/-}$ (●) mice in response to 20 mmol/l arginine and 50 μmol/l carbachol is shown. *D*: Changes in serum insulin levels in response to mixed meal ingestion. Serum insulin levels in Kir6.2$^{+/+}$ (□, n = 15) and Kir6.2$^{-/-}$ (■, n = 15) mice at indicated time points are shown. *P < 0.005. *E*: Changes in blood glucose levels in response to mixed meal ingestion. Blood glucose levels in Kir6.2$^{+/+}$ (○, n = 15) and Kir6.2$^{-/-}$ (●, n = 15) mice are shown. *P < 0.0001.

(Fig. 2*B*), we considered the possibility that GIP inhibits gut motility in a K_{ATP} channel–dependent manner, but this apparently is not the case (Fig. 2*C*). This is compatible with a recent study of GIP action on gut motility in humans (34). We also found a significant increase in gastrointestinal transit by oral glucose load. We established previously that K_{ATP} channels comprising Kir6.2 and SUR1 are found in glucose-responsive neurons in the hypothalamus (35) and in the ileum (36), and we proposed that the K_{ATP} channel in gut cholinergic neurons plays a role in glucose-evoked reflexes (36). Ingestion of carbohydrate is known to stimulate gastrointestinal motility (37), but it was unclear whether the K_{ATP} channel in glucose-responsive enteric neurons is involved in regulating glucose-induced gut motility. Our present findings on gastrointestinal transit in Kir6.2$^{-/-}$ mice clearly show that gut motility is not regulated by K_{ATP} channel–mediated glucose sensing in the enteric neurons (Fig. 2*D*).

Measurement of serum insulin at 10 min after oral glucose load revealed that GIP pretreatment in vivo failed to potentiate the early-phase (38) insulin secretion during OGTT in Kir6.2$^{-/-}$ mice (Fig. 3), indicating that the K_{ATP} channel in β-cells is essential in the insulinotropic effect of GIP. It would be likely, therefore, that the glucose-dependent effects of GIP depend on the activity of the K_{ATP} channel. In contrast, there was significant potentiation of late-phase insulin secretion (2.17-fold increase) in Kir6.2$^{-/-}$ mice by GIP pretreatment. However, the physiological significance of this late-phase insulin secretion remains uncertain, because there was no significant reduction in blood glucose levels even after 30 min in GIP-pretreated Kir6.2$^{-/-}$ mice compared with GIP-untreated Kir6.2$^{-/-}$ mice. These results also suggest that rapid enhancement of early-phase insulin secretion by GIP is required for its glucose-lowering effect after oral glucose load.

In contrast to GIP, GLP-1 did potentiate the insulin secretion (3.7-fold increase in 10 min) and had an obvious antihyperglycemic effect in Kir6.2$^{-/-}$ mice (Fig. 4*A*, *B*, and *D*). Perfusion experiments of mouse pancreata are applicable only for a short period (less than 45 min of sampling) of secretion study of insulin. Thus, it is difficult to perform multiple stimuli in the same mouse pancreas, and a number of experiments are required to compare the secretory differences among different stimuli. However, when compared with the study of isolated islets, this method has an advantage because we can neglect cellular damage during islet isolation or unexpected effects by culturing the islets.

We performed perfusion experiments in Kir6.2$^{-/-}$ mice and found that differences in the insulinotropic effects between GIP and GLP-1 in Kir6.2$^{-/-}$ mice were also shown in the perfusion experiments (Fig. 5). Accordingly, the mechanism of potentiation of insulin secretion differs for GIP and GLP-1: insulin secretion by GIP depends critically on the K_{ATP} channel, whereas that by GLP-1 does not. Both

GIP and GLP-1 increase the intracellular cAMP concentration and potentiate insulin secretion by activating protein kinase A (PKA)– and/or cAMP-GEF2–mediated signaling in normal pancreatic β-cells (39). We previously reported that GIP-potentiated insulin secretion is almost completely suppressed in islets treated both with PKA blocker H-89 and antisense oligodeoxynucleotides against cAMP-GEF2, whereas GLP-1–potentiated insulin secretion remains nearly normal (15). Apparently, the insulinotropic action of GLP-1 is mediated by a pathway other than that involving PKA and cAMP-GEF2. In addition, we found that whereas GIP had almost no effect on gut motility, GLP-1 significantly suppressed gastrointestinal transit (Fig. 3*C*). Because the effect of GLP-1 is independent of the K_{ATP} channels, GLP-1 may well delay glucose absorption and prevent a rise in blood glucose levels after glucose load in Kir6.2$^{-/-}$ mice. Thus, GLP-1 is suggested to participate in the postprandial glycemic control in K_{ATP} channel–independent manners by potentiating insulin secretion and by delaying gastric emptying. Although the importance of the K_{ATP} channel in the potentiation of insulin secretion by cAMP has been shown in SUR1 knockout mice (24,40), we clarify here the involvement of the channel in the potentiation of insulin secretion by GIP and GLP-1.

Although arginine treatment elicited impaired insulin secretion, the insulin secretion of Kir6.2$^{-/-}$ mice in response to carbachol was intact, indicating that the exocytotic machinery of Kir6.2$^{-/-}$ β-cells is intact and that the cause of impaired insulin secretion differs according to the stimulus (Fig. 6*A–C*). Insulin secretion is stimulated by multiple signals in pancreatic β-cells, including nutrients (carbohydrate, proteins, and fat), incretins (GIP and GLP-1), and neuronal input (mainly cholinergic). Our results indicate that mice lacking the Kir6.2 pore-forming subunit of K_{ATP} channels have an impaired insulin secretory response to glucose, arginine, and GIP, whereas the insulin secretion elicited by carbachol is comparable with that in Kir6.2$^{+/+}$ mice. Kir6.2$^{-/-}$ mice were also shown to exhibit glucose intolerance and delayed insulin secretion in response to mixed meal (Fig. 6*C* and *D*). The K_{ATP} channel thus plays an important role in regulating blood glucose levels both after glucose load and after ingestion of a mixed meal. The present study shows that the K_{ATP} channel in pancreatic β-cells is required for the insulinotropic effects of GIP through the potentiation of glucose-induced insulin secretion. In contrast, the potentiation of insulin secretion by GLP-1 depends on K_{ATP} channel–independent and –dependent mechanisms. The differing pathways of the action of GLP-1 and GIP on both the potentiation of insulin and gut motility might well account for the differences seen in their therapeutic efficacy in type 2 diabetes.

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REFERENCES

1. Elrick H, Stimmler L, Hlad CJ, Arai Y: Plasma insulin response to oral and intravenous glucose administration. *J Clin Endocrinol Metab* 24:1076–1082, 1964
2. Perley MJ, Kipnis DM: Plasma insulin responses to oral and intravenous glucose: studies in normal and diabetic subjects. *J Clin Invest* 46:1954–1962, 1967
3. Drucker DJ: Minireview: the glucagon-like peptides. *Endocrinology* 142: 521–527, 2001
4. Meier JJ, Nauck MA, Schmidt WE, Gallwitz B: Gastric inhibitory polypeptide: the neglected incretin revisited. *Regul Pept* 107:1–13, 2002
5. Elliott RM, Morgan LM, Tredger JA, Deacon S, Wright J, Marks V: Glucagon-like peptide-1 (7–36) amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: acute post-prandial and 24-h secretion patterns. *J Endocrinol* 138:159–166, 1993
6. Dupre J, Ross SA, Watson D, Brown JC: Stimulation of insulin secretion by gastric inhibitory polypeptide in man. *J Clin Endocrinol Metab* 37:826–828, 1973
7. Pederson RA, Schubert HE, Brown JC: Gastric inhibitory polypeptide: its physiologic release and insulinotropic action in the dog. *Diabetes* 24:1050–1056, 1975
8. Holst JJ, Orskov C, Nielsen OV, Schwartz TW: Truncated glucagon-like peptide I, an insulin-releasing hormone from the distal gut. *FEBS Lett* 211:169–174, 1987
9. Kreymann B, Williams G, Ghatei MA, Bloom SR: Glucagon-like peptide-1 7–36: a physiological incretin in man. *Lancet* 2:1300–1304, 1987
10. Usdin TB, Mezey E, Button DC, Brownstein MJ, Bonner TI: Gastric inhibitory polypeptide receptor, a member of the secretin-vasoactive intestinal peptide receptor family, is widely distributed in peripheral organs and the brain. *Endocrinology* 133:2861–2870, 1993
11. Thorens B: Expression cloning of the pancreatic beta cell receptor for the gluco-incretin hormone glucagon-like peptide 1. *Proc Natl Acad Sci U S A* 89:8641–8645, 1992
12. Szecowka J, Grill V, Sandberg E, Efendic S: Effect of GIP on the secretion of insulin and somatostatin and the accumulation of cyclic AMP in vitro in the rat. *Acta Endocrinol (Copenh)* 99:416–421, 1982
13. Drucker DJ, Philippe J, Mojsov S, Chick WL, Habener JF: Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *Proc Natl Acad Sci U S A* 84:3434–3438, 1987
14. Fehmann HC, Goke R, Goke B: Cell and molecular biology of the incretin hormones glucagon-like peptide-I and glucose-dependent insulin releasing polypeptide. *Endocr Rev* 16:390–410, 1995
15. Kashima Y, Miki T, Shibasaki T, Ozaki N, Miyazaki M, Yano H, Seino S: Critical role of cAMP-GEFII-Rim2 complex in incretin-potentiated insulin secretion. *J Biol Chem* 276:46046–46053, 2001
16. Nauck MA, Heimesaat MM, Orskov C, Holst JJ, Ebert R, Creutzfeldt W: Preserved incretin activity of glucagon-like peptide 1 [7–36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus. *J Clin Invest* 91:301–307, 1993
17. Miyawaki K, Yamada Y, Yano H, Niwa H, Ban N, Ihara Y, Kubota A, Fujimoto S, Kajikawa M, Kuroe A, Tsuda K, Hashimoto H, Yamashita T, Jomori T, Tashiro F, Miyazaki J, Seino Y: Glucose intolerance caused by a defect in the entero-insular axis: a study in gastric inhibitory polypeptide receptor knockout mice. *Proc Natl Acad Sci U S A* 96:14843–14847, 1999
18. Pederson RA, Brown JC: Interaction of gastric inhibitory polypeptide, glucose, and arginine on insulin and glucagon secretion from the perfused rat pancreas. *Endocrinology* 103:610–615, 1978
19. Nauck MA, Homberger E, Siegel EG, Allen RC, Eaton RP, Ebert R, Creutzfeldt W: Incretin effects of increasing glucose loads in man calculated from venous insulin and C-peptide responses. *J Clin Endocrinol Metab* 63:492–498, 1986
20. Elahi D, Andersen DK, Brown JC, Debas HT, Hershcopf RJ, Raizes GS, Tobin JD, Andres R: Pancreatic alpha- and beta-cell responses to GIP infusion in normal man. *Am J Physiol* 237:E185–E191, 1979
21. Nauck MA, Bartels E, Orskov C, Ebert R, Creutzfeldt W: Additive insulinotropic effects of exogenous synthetic human gastric inhibitory polypeptide and glucagon-like peptide-1-(7–36) amide infused at near-physiological insulinotropic hormone and glucose concentrations. *J Clin Endocrinol Metab* 76:912–917, 1993
22. Miki T, Nagashima K, Tashiro F, Kotake K, Yoshitomi H, Tamamoto A,

Gonoi T, Iwanaga T, Miyazaki J, Seino S: Defective insulin secretion and enhanced insulin action in KATP channel-deficient mice. *Proc Natl Acad Sci U S A* 95:10402–10406, 1998
23. Seghers V, Nakazaki M, DeMayo F, Aguilar-Bryan L, Bryan J: Surl knockout mice: a model for K(ATP) channel-independent regulation of insulin secretion. *J Biol Chem* 275:9270–9277, 2000
24. Shiota C, Larsson O, Shelton KD, Shiota M, Efanov AM, Hoy M, Lindner J, Kooptiwut S, Juntti-Berggren L, Gromada J, Berggren PO, Magnuson MA: Sulfonylurea receptor type 1 knock-out mice have intact feeding-stimulated insulin secretion despite marked impairment in their response to glucose. *J Biol Chem* 277:37176–37183, 2002
25. Krarup T, Madsbad S, Moody AJ, Regeur L, Faber OK, Holst JJ, Sestoft L: Diminished gastric inhibitory polypeptide (GIP) response to a meal in newly diagnosed type 1 (insulin dependent) diabetics. *J Clin Endocrinol Metab* 56:1306–1312, 1983
26. Deacon CF, Nauck MA, Meier J, Hucking K, Holst JJ: Degradation of endogenous and exogenous gastric inhibitory polypeptide in healthy and in type 2 diabetic subjects as revealed using a new assay for the intact peptide. *J Clin Endocrinol Metab* 85:3575–3581, 2000
27. Miki T, Tashiro F, Iwanaga T, Nagashima K, Yoshitomi H, Aihara H, Nitta Y, Gonoi T, Inagaki N, Miyazaki J, Seino S: Abnormalities of pancreatic islets by targeted expression of a dominant-negative K_{ATP} channel. *Proc Natl Acad Sci U S A* 94:11969–11973, 1997
28. Bonnevie-Nielsen V, Steffes MW, Lernmark A: A major loss in islet mass and B-cell function precedes hyperglycemia in mice given multiple low doses of streptozotocin. *Diabetes* 30:424–429, 1981
29. Guenifi A, Ahren B, Abdel-Halim SM: Differential effects of glucagon-like peptide-1 (7–36)amide versus cholecystokinin on arginine-induced islet hormone release in vivo and in vitro. *Pancreas* 22:58–64, 2001
30. Havel PJ, Akpan JO, Curry DL, Stern JS, Gingerich RL, Ahren B: Autonomic control of pancreatic polypeptide and glucagon secretion during neuroglucopenia and hypoglycemia in mice. *Am J Physiol* 265:R246–R254, 1993
31. Scrocchi LA, Brown TJ, MaClusky N, Brubaker PL, Auerbach AB, Joyner AL, Drucker DJ: Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide 1 receptor gene. *Nat Med* 2:1254–1258, 1996
32. Buffa R, Polak JM, Pearse AG, Solcia E, Grimelius L, Capella C: Identification of the intestinal cell storing gastric inhibitory peptide. *Histochemistry* 43:249–255, 1975
33. Cataland S, Crockett SE, Brown JC, Mazzaferri EL: Gastric inhibitory polypeptide (GIP) stimulation by oral glucose in man. *J Clin Endocrinol Metab* 39:223–228, 1974
34. Meier JJ, Goetze O, Anstipp J, Hagemann D, Holst JJ, Schmidt WE, Gallwitz B, Nauck MA: Gastric inhibitory polypeptide does not inhibit gastric emptying in humans. *Am J Physiol Endocrinol Metab* 286:E621–E625, 2004
35. Miki T, Liss B, Minami K, Shiuchi T, Saraya A, Kashima Y, Horiuchi M, Ashcroft F, Minokoshi Y, Roeper J, Seino S: ATP-sensitive K^+ channels in the hypothalamus are essential for the maintenance of glucose homeostasis. *Nat Neurosci* 4:507–512, 2001
36. Liu M, Seino S, Kirchgessner AL: Identification and characterization of glucoresponsive neurons in the enteric nervous system. *J Neurosci* 19:10305–10317, 1999
37. Rao SS, Kavelock R, Beaty J, Ackerson K, Stumbo P: Effects of fat and carbohydrate meals on colonic motor response. *Gut* 46:205–211, 2000
38. Lewis JT, Dayanandan B, Habener JF, Kieffer TJ: Glucose-dependent insulinotropic polypeptide confers early phase insulin release to oral glucose in rats: demonstration by a receptor antagonist. *Endocrinology* 141:3710–3716, 2000
39. Ozaki N, Shibasaki T, Kashima Y, Miki T, Takahashi K, Ueno H, Sunaga Y, Yano H, Matsuura Y, Iwanaga T, Takai Y, Seino S: cAMP-GEFII is a direct target of cAMP in regulated exocytosis. *Nat Cell Biol* 2:805–811, 2000
40. Nakazaki M, Crane A, Hu M, Seghers V, Ullrich S, Aguilar-Bryan L, Bryan J: cAMP-activated protein kinase-independent potentiation of insulin secretion by cAMP is impaired in SUR1 null islets. *Diabetes* 51:3440–3449, 2002